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Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: An FAD- and FMN-containing flavoprotein

(L-arginine/endothelium-derived relaxing factor/interferon γ)

DENNIS J. STUEHR*†, HEARN J. CHO, NYOUN SOO KWON‡, MARY F. WEISE, AND CARL F. NATHAN*

The Beatrice and Samuel A. Seaver Laboratory, Division of Hematology–Oncology, Department of Medicine, Cornell University Medical College, New York, NY 10021

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ABSTRACT **A soluble nitric oxide (NO) synthase activity was purified 426-fold from a mouse macrophage cell line activated with interferon γ and bacterial lipopolysaccharide by sequential anion-exchange, affinity, and gel filtration chromatography. SDS/PAGE of the purified NO synthase gave three closely spaced silver-staining protein bands between 125 and 135 kDa. When assayed in the presence of L-arginine, NADPH, tetrahydrobiopterin, FAD, and reduced thiol, purified NO synthase had a specific activity of 1313 nmol of NO_2^- plus NO_3^- per min per mg. The apparent K_m of the enzyme for L-arginine and NADPH was 2.8 and 0.3 μM, respectively. Addition of calcium ions with or without calmodulin did not increase the activity of the purified enzyme, and NO synthesis was not altered by calmodulin inhibitors. Gel filtration chromatography indicated that the induced NO synthase was catalytically competent as a dimer of ≈250 kDa but could be dissociated into inactive monomers of ≈130 kDa in the absence of L-arginine, FAD, and tetrahydrobiopterin. Upon heat denaturation, NO synthase released 1.1 mol of FAD and 0.55 mol of FMN per mol of 130-kDa subunit. Thus, inducible macrophage NO synthase differs in several respects from constitutive NO synthases and is one of very few eukaryotic enzymes containing both FAD and FMN.**

The free radical nitric oxide (NO) or a NO-releasing product is synthesized within mammalian immune, cardiovascular, and neural systems, where it functions as a signaling or cytotoxic molecule (for reviews, see refs. 1–3). The mammalian enzymes that generate NO are not completely characterized. Current evidence suggests that there are at least two forms. One is constitutively expressed, requires calcium ions and a calcium-binding protein such as calmodulin for its activation, and participates in signal transduction by generating NO in response to increased intracellular calcium levels, leading to activation of soluble guanylyl cyclase by NO (1, 4–7). The other form is expressed in cells only after several hours of exposure to cytokines like interferon γ (IFN-γ) and/or microbial products such as bacterial lipopolysaccharide (LPS) (8–10). Immunologically induced NO synthase participates in the destruction of microbial pathogens and tumor cells and contributes to shock associated with sepsis (2, 3, 11, 12). The inducible NO synthase appears to be antigenically distinct from constitutive NO synthase (13).

Despite their differences in regulation and function, evidence suggests that the constitutive and induced NO synthases are catalytically similar. Both types utilize NADPH and, where it was tested, tetrahydrobiopterin as redox cofactors (4–7, 14, 15) and convert L-arginine to NO and L-citrulline with one atom of molecular oxygen being incorporated into L-citrulline (16). In cases where it was tested, both constitutive and inducible activities were enhanced by exogenous FAD (7, 17) and inhibited irreversibly by the flavoprotein inhibitor diphenyleneiodonium (18), suggesting that NO synthases may be flavoproteins. However, a direct demonstration that the NO synthases contain flavins has not been obtained.

Thus far only constitutive NO synthases have been purified to homogeneity, from rat and porcine cerebellum (4–6) and rat neutrophils (7). In this report we describe the purification of an IFN-γ- and LPS-induced NO synthase from a mouse macrophage cell line and show that it is a flavoprotein containing not only FAD but also FMN.

MATERIALS AND METHODS

Dithiothreitol was from Pierce. Adenosine 2′,5′-bisphosphate (2′,5′-ADP)-Sepharose resin and prepacked Mono Q HR 10/10, TSK G3000 SW (7.5 × 600 mm), and TSK G4000 SW (7.5 × 600 mm) columns were from Pharmacia LKB. All other reagents were obtained from Sigma or from sources as previously reported (14, 16, 17). Recombinant mouse IFN-γ was a gift of Genentech.

Purification of NO Synthase. RAW 264.7 macrophages were grown at 37°C, 5% CO_2 in 6 liters of RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 8% bovine calf serum. When the cells reached a density of ≈10^6 cells per ml, IFN-γ (100 units/ml) and *Escherichia coli* LPS (2 μg/ml) were added to induce NO synthase activity. After 10–12 hr, the cells were harvested by centrifugation at 4°C and resuspended in 80 ml of ice-cold saline that contained 25 mM glucose, typically yielding about 5 × 10^9 cells with a viability (by trypan-blue exclusion) of >90%. The cells were repelleted and resuspended in 16 ml of cold H_2O containing protease inhibitors as described previously (17), then lysed by three cycles of rapid freeze–thawing. EGTA and EDTA were omitted from the lysis buffer because they inhibit NO synthase activity in the crude lysate (19). The lysate was centrifuged at 100,000 × *g* for 90 min at 4°C and the supernatant was stored at −80°C.

All chromatography for purification was carried out on a Pharmacia FPLC instrument at room temperature, and eluent fractions were collected into plastic tubes kept on ice. Buffer for all purification steps was 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (pH 7.2) containing 5 mM L-arginine, 3 mM dithiothreitol, 2 μM FAD, 1 μM tetrahydrobiopterin [(6*RS*)-2-amino-4-hydroxy-6-(L-*erythro*-1,2-

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Abbreviations: IFN-γ, interferon γ; LPS, lipopolysaccharide.

*To whom reprint requests should be addressed.
†Present address: Immunology Section, NN-1, The Cleveland Clinic, Cleveland, OH 44197.
‡Present address: Department of Biochemistry, Chung-Ang University Medical College, Dongjakgu, Seoul, 156-756, South Korea.

dihydroxypropyl)-5,6,7,8-tetrahydropteridine], and 10% (vol/vol) glycerol unless specified otherwise. The active cell supernatant was chromatographed in three runs of 5 ml each on a Mono Q column at a flow rate of 2 ml/min. A programmed gradient was run from 0.12 to 1.0 M NaCl to elute NO synthase activity (Fig. 1*A*). Active fractions (≈10 ml for each run) were loaded directly at 0.3 ml/min onto a 5 × 100-mm column containing 2′,5′-ADP-Sepharose. After unbound protein had been eluted, the nonspecifically bound proteins were eluted with 5 ml of buffer containing 0.6 M NaCl. NO synthase was then eluted with 5 ml of buffer containing 8 mM NADPH (Fig. 1*B*). The active fractions from three runs were pooled and concentrated at 4°C in a Centricon-30 microconcentrator (Amicon). The concentrate was washed twice with 1 ml of buffer to remove most of the residual NADPH, and the sample (300–400 μl) was stored at −80°C. Gel filtration on TSK G3000 or G4000 SW columns was carried out at 0.25 ml/min on 50-μl aliquots of concentrated sample, using column buffer supplemented with 0.2 M NaCl. Protein was eluted in two peaks, the first being NO synthase, which was obtained in ≈1.5 ml (Fig. 1*C*). Purified

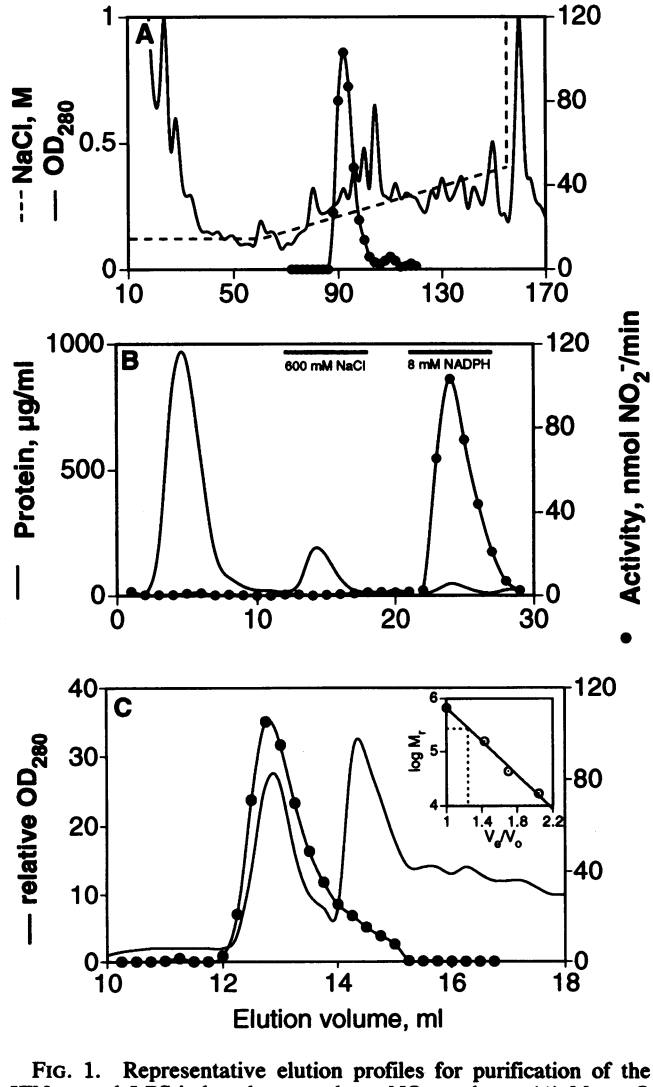

FIG. 1. Representative elution profiles for purification of the IFN-γ- and LPS-induced macrophage NO synthase. (*A*) Mono Q anion-exchange chromatography of the induced macrophage supernatant. (*B*) 2′,5′-ADP-Sepharose affinity chromatography of Mono Q active fractions. (*C*) Size-exclusion chromatography of concentrated NO synthase activity from *B* on a TSK G3000 SW column. Molecular weight standards shown in the *Inset* are bovine thyroglobulin (670,000), bovine γ-globulin (158,000), ovalbumin (44,000), and horse myoglobin (17,000).

enzyme could be stored at −80°C. For certain experiments, gel filtration was performed in buffer without L-arginine or without FAD and tetrahydrobiopterin.

Assay Conditions. NO synthase activity was assayed by diluting 10 μl of each column fraction in 90 μl of 40 mM Tris·HCl buffer (pH 7.9), containing 2 mM NADPH, 4 μM tetrahydrobiopterin, 4 μM FAD, 3 mM dithiothreitol, and 1 mM L-arginine. Final assay pH was 7.82 ± 0.02. Fractions were assayed in duplicate. Reactions were initiated by addition of NADPH. After 1.5 hr at 37°C, residual NADPH was removed enzymatically and nitrite was assayed colorimetrically as described (10, 17).

Kinetic Measurements. The initial rate of NO synthesis was measured spectrophotometrically using the oxyhemoglobin assay for NO (20) as recently described (21). In some cases, production of nitrite (NO_2^-) and nitrate (NO_3^-), stable oxidation products of NO that accumulate quantitatively over time, was monitored by an automated nitrite/nitrate analyzer as described (17).

Measurement of FAD and FMN Released from NO Synthase. NO synthase was purified as above except that the gel filtration step was performed in the absence of FAD. A portion of purified NO synthase (8 μg, 1 ml) was boiled for 7 min to release noncovalently bound FAD and FMN, and the sample was deproteinated by filtration through Centricon-30 microconcentrators and stored on ice. FAD and FMN in 0.3-ml aliquots of the sample were separated by HPLC on an Applied Biosystems/Brownlee Lab RP-18 reverse-phase column (220 × 4.6 mm) by a published procedure (22) with modifications. Briefly, the conditions at injection were 93% buffer A (5 mM ammonium acetate, pH 6.0)/7% methanol, flowing at 1 ml/min. After 2 min, a linear gradient was developed over 13 min to 70% methanol. FAD and FMN were detected using a Hitachi S-1000 flow-through fluorometer set at 460 nm for excitation and 530 nm for emission. Under these conditions, authentic FAD and FMN were completely resolved and eluted at 11.2 and 13.1 min, respectively. FAD and FMN in NO synthase samples were quantitated by measuring peak heights relative to standard curves, which were linear (correlation coefficient, $r = 0.99$) between 0 and 45 pmol of FAD or FMN. The HPLC-purified fluorophores released from NO synthase were collected separately and their excitation–emission spectra were obtained using a Spex Industries (Edison, NJ) Fluorolog fluorometer. Spectra obtained were compared with those of authentic FAD and FMN dissolved in the same HPLC buffer.

Protein Determination. Protein was determined by the method of Bradford (23) using the Bio-Rad assay solution and bovine serum albumin as a standard.

RESULTS

Induced NO synthase was purified 426-fold from the soluble fraction of IFN-γ- and LPS-treated RAW 264.7 cells by a three-step procedure as illustrated in Fig. 1 and Table 1. The final specific activity was 1313 nmol of NO_2^- plus NO_3^- produced per mg of enzyme per min, with an overall recovery of 9%. The greatest individual purification factor (9.2-fold) was achieved by affinity chromatography on 2′,5′-ADP-Sepharose, a step introduced for partial purification of macrophage NO synthase (24) that has been used in all subsequent purifications of the constitutive NO synthases (4–7). The SDS/PAGE profile at each purification step is illustrated in Fig. 2; the purified NO synthase is a tight triplet of silver-stained protein bands of estimated molecular mass 125–135 kDa (lane D). Noninduced RAW 264.7 cell supernatant purified in an identical manner through the Mono Q and 2′,5′-ADP-Sepharose steps did not contain proteins in this mass region (lane E). This may indicate that the protein bands associated with NO synthase were induced by IFN-

Table 1. Purification of cytokine-induced macrophage NO synthase

Fraction	Protein, mg	Total activity*	Specific activity†	Yield, %	Purification factor
Lysate sup.‡	198	487.2	2.5	100	1
Mono Q	7.6	141.2	21.3	29	9
2',5'-ADP	0.27	50.0	197	10.2	83
TSK G3000	0.04	42.4	1060	8.7	426

RAW 264.7 cells were harvested after 10–12 hr of incubation with IFN-γ and LPS. Values are averages from three purifications, starting with a mean of 5×10^9 cells.
*nmol of NO_2^- per min.
†nmol of NO_2^- per min per mg of protein (NO_3^- was not measured).
‡Supernatant of 100,000 × *g* centrifugation of cell lysate.

γ/LPS. The molecular mass obtained for NO synthase by SDS/PAGE was about half of that estimated for active NO synthase (250 kDa) on both TSK G3000SW and TSK G4000SW gel filtration columns (Fig. 1*C Inset*).

The column buffers used for enzyme purification were supplemented with 3 mM DTT, 5 mM L-arginine, 1 μM tetrahydrobiopterin, 2 μM FAD, and 10% glycerol to maximize yield of active enzyme. Gel filtration of NO synthase under these conditions resulted in 85% recovery of enzyme activity (Table 1) and could be performed in the absence of L-arginine without further loss (data not shown). However, when the gel filtration step was performed in the absence of L-arginine, tetrahydrobiopterin, and FAD, recovery of NO synthase activity fell to 15% (Fig. 3). Also, the retention time of the NO synthase protein peak increased such that its estimated molecular mass decreased from 250 kDa to 121 kDa (Fig. 3 *Inset*). This change was reflected in analytical SDS/PAGE, which showed the 130-kDa proteins eluted in the fractions corresponding to the lower molecular mass (data not shown). When the same column buffer was made 5 mM in L-arginine and a new sample was run under otherwise identical conditions, both retention time and recovery of activity were restored to their normal values (Fig. 3). The results suggest that either L-arginine alone or a combination of FAD and tetrahydrobiopterin is sufficient to maintain the enzyme in its larger, active form. However, as evident in Fig. 3, cofactors added after the dimer had been dissociated did not restore enzymatic activity.

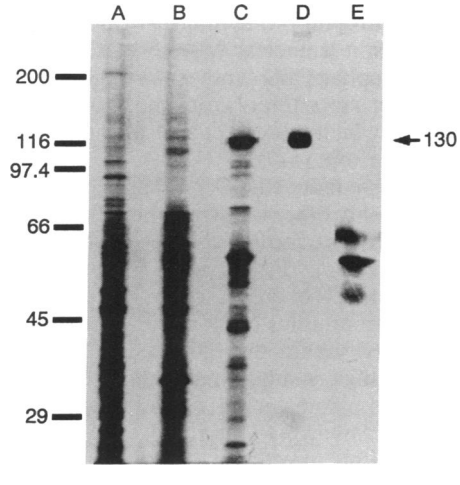

FIG. 2. Representative SDS/PAGE profile of NO synthase activity at each purification step (7.5% acrylamide gel, 1 μg of protein per lane, silver-stained). Lane A, crude lysate; lane B, after Mono Q step; lane C, after 2',5'-ADP-Sepharose step; lane D, after TSK G3000 SW step; lane E, noninduced-macrophage lysate supernatant chromatographed through the Mono Q and 2',5'-ADP-Sepharose steps. Molecular weight standards indicated are rabbit muscle myosin heavy chain (200,000), *E. coli* β-galactosidase (116,000), rabbit muscle phosphorylase *b* (97,400), bovine albumin (66,000), ovalbumin (45,000), and bovine carbonic anhydrase (29,000).

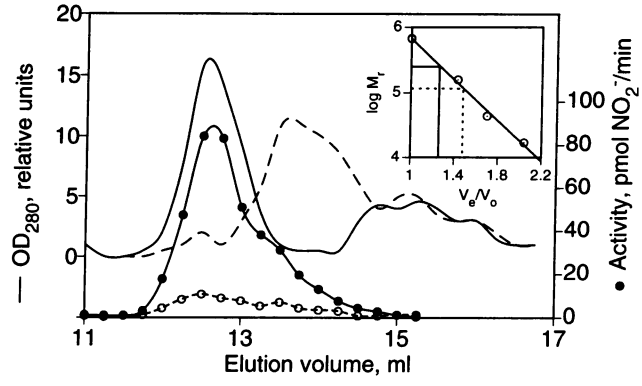

FIG. 3. Effect of buffer conditions on the elution and stability of NO synthase during TSK gel filtration. NO synthase was chromatographed on a TSK G3000 SW column in the absence of L-arginine, FAD, and tetrahydrobiopterin (dashed line, OD_{280}; activity, open symbols), or in the absence of only FAD and tetrahydrobiopterin (solid line, OD_{280}; activity, filled symbols). (*Inset*) Protein peak molecular weight relative to molecular weight standards during chromatography with L-arginine (solid line) and without L-arginine (dashed line).

The purified NO synthase activity could be stored unconcentrated at ≈15 μg/ml for at least 6 hr at 4°C or overnight at −80°C without loss of activity. Under our standard assay conditions, the specific activity of NO synthase remained constant with dilution over the protein concentration range tested (0.05–1.3 μg/ml). When NO synthase was incubated at 37°C under assay conditions, the specific activity decreased slowly, falling to 56% of its original value after 6 hr (data not shown).

Some physical and kinetic parameters of purified macrophage NO synthase are listed in Table 2. The evidence thus far suggests that macrophage NO synthase is active as a dimer comprised of two ≈130-kDa subunits. The K_m for L-arginine as substrate (2.8 μM) was similar to the value obtained previously (2.3 μM) for partially purified enzyme (21). The K_m for NADPH was estimated to be 0.3 μM.

Upon heat denaturation, NO synthase released two fluorophores whose retention times on reverse-phase HPLC matched those of authentic FAD and FMN (Fig. 4). (Authentic FAD boiled under these conditions was stable and did not generate FMN.) The peaks were collected separately and their fluorescence excitation–emission spectra were recorded. Both peaks had excitation maxima at 280, 365, and 460 nm and emission maxima at 430 and 530 nm, identical to authentic FAD and FMN. NO synthase released an average of 1.1 ± 0.1 mol of FAD and 0.55 ± 0.04 mol of FMN per mol of 130-kDa subunit ($n = 2$).

Added FAD, tetrahydrobiopterin, and FMN each enhanced NO synthesis by the purified enzyme at submicromolar con-

Table 2. Physical and kinetic characteristics of cytokine-induced macrophage NO synthase

Molecular mass	
Native	250 kDa
Denatured	125–135 kDa
V_{max}	1.3 μmol of NO_2^- plus NO_3^- per min per mg
K_m, L-arginine	2.8 μM
K_m, NADPH	≈0.3 μM
FAD content	1.1 ± 0.1 per 130 kDa
FMN content	0.55 ± 0.04 per 130 kDa

Native molecular mass was estimated by gel filtration on TSK G3000 SW and G4000 SW columns. Denatured molecular weight was estimated by SDS/PAGE.

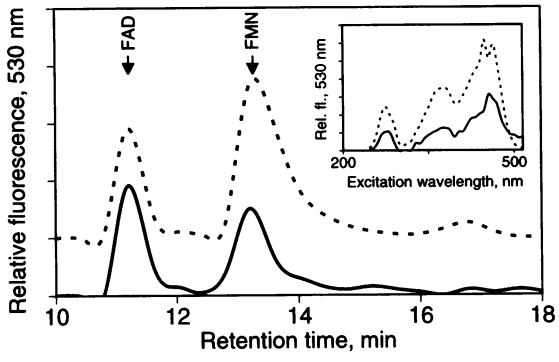

FIG. 4. Reverse-phase HPLC analysis of fluorophores released from 2.9 μg (22.3 pmol of monomer) of boiled NO synthase (solid line) compared with FAD and FMN standards (dashed line). Retention times for authentic FAD (2.4 nmol) and FMN (2.2 nmol) were 11.2 and 13.1 min, respectively. The illustration is representative of two analyses. (*Inset*) Fluorescent emission spectrum of authentic FMN (dashed line) compared with that of the NO synthase-derived fluorophore eluted at 13 min (solid line).

centrations (Table 3). Maximal stimulation required at least 1 μM concentration of each. The rate of NO synthesis was completely dependent on NADPH (data not shown) and partially dependent on added tetrahydrobiopterin, FAD, and FMN (Table 3). In contrast to the linear rates observed in the absence of FAD or FMN, the rate in the absence of tetrahydrobiopterin fell continuously and became essentially zero after 90 min. These results are similar to those reported for the partially purified induced NO synthase (17).

The activity of constitutive NO synthases is highly dependent on added calcium ions (7) or calcium ions plus calmodulin (4–6) and is potently inhibited by drugs directed against calcium-binding proteins. In contrast, induced NO synthase activity was enhanced <20% by added calcium (2 mM) or calcium plus calmodulin (100 units/ml), and four calmodulin inhibitors did not decrease crude cytosolic NO synthase activity appreciably (Table 4). Addition of EDTA or EGTA at 1 mM to Mono Q → 2′,5′-ADP-Sepharose-purified preparations of induced enzyme did not inhibit NO synthase activity (data not shown).

DISCUSSION

Cytokine induction of NO synthase activity was first described in mouse macrophages (8, 9) and has since been

Table 3. Cofactor dependency of the cytokine-induced NO synthase

Cofactor omitted	Rate of NO synthesis in the absence of cofactor, pmol/min	EC_{50},* nM
None	541	
FMN	454	100
FAD	154	40
H_4biopterin†		
0–25 min	220	60
60–90 min	55	

For this experiment, gel filtration was performed in the absence of FAD, FMN, and tetrahydrobiopterin (H_4biopterin). Nitrite and nitrate production over time was measured in reaction mixtures that contained all cofactors at optimal concentrations (control) or in reaction mixtures that were missing the single designated cofactor.
*The cofactor concentration that supported a rate of NO synthesis that was midway between the rate of a fully supplemented reaction and the rate in a reaction missing the cofactor.
†The rate of NO synthesis in the absence of H_4biopterin continuously decreased. The reported rates are the average within the two time frames noted.

Table 4. Effect of calcium ions, calmodulin, and calmodulin inhibitors on induced NO synthase activity

Additive(s)	Purified NO synthase activity*	Crude NO synthase activity†
None	203 ± 13	340 ± 4
Ca^{2+} (2 mM)	241 ± 5	
Ca^{2+}/calmodulin (100 units/ml)	224 ± 16	
Trifluoperazine (10 μM)		311 ± 17
N6 (100 μM)		313 ± 81
N5 (100 μM)		314 ± 19
N4 (100 μM)		337 ± 21

Reaction mixtures contained purified NO synthase or unfractionated macrophage supernatant (crude NO synthase) and were incubated for 3 hr under the standard assay conditions in the presence or absence of the listed additives: N6, *N*-(6-aminohexyl)-5-chloro-2-naphthalenesulfonamide; N5, *N*-(5-aminopentyl)-1-naphthalenesulfonamide; N4, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.
*nmol of NO_2^- per min per mg.
†nmol of NO_2^- plus NO_3^- per min.

observed in a variety of tissues and cells (refs. 25–28, reviewed in ref. 19). We have purified the IFN-γ- and LPS-induced NO synthase from a mouse macrophage cell line and found that it is an FAD- and FMN-containing flavoprotein. Our evidence suggests that this induced macrophage NO synthase differs from the constitutive cerebellar or neutrophil NO synthases in at least four ways. (*i*) Induced NO synthase subunits are somewhat heterogeneous with regard to size (ranging from 125 to 135 kDa) and are slightly smaller than the SDS-denatured constitutive NO synthases of rat cerebellum (150 or 155 kDa; refs. 4 and 5), porcine cerebellum (160 kDa; ref. 6), or rat neutrophils (150 kDa; ref. 7). (*ii*) Macrophage NO synthase appears to be catalytically active as a dimer, whereas three of four constitutive NO synthases purified thus far were reported to be active as monomers (4, 6, 7). (*iii*) The induced macrophage enzyme does not require added calcium ions or calmodulin for activity. (*iv*) The induced synthase is much more stable at 4°C or when undergoing catalysis at 37°C than either the cerebellar or the neutrophil NO synthases. Due to differences in assay conditions, it is unclear whether the maximal velocity of induced macrophage NO synthase (≈1.3 μmol of NO_2^- plus NO_3^- per mg per min) differs from those of the constitutive NO synthases, which were reported to range from 0.1 to 1 μmol per mg per min (4–7).

It is unusual that induced NO synthase contains both FAD and FMN. The only other known mammalian enzyme in this class is NADPH–cytochrome P-450 oxidoreductase, which contains 1 mol each of FAD and FMN per mol of enzyme (29). Its FAD and FMN cofactors facilitate the one-electron reduction of hemoproteins by stabilizing the resultant radical that is generated within the oxidoreductase (30, 31). NO synthase may utilize a similar mechanism during generation of NO, which is paramagnetic, from the initial diamagnetic intermediate N^{ω}-hydroxy-L-arginine (19, 21). In addition, NO synthase is an oxygenase (16, 21) and therefore may share some properties with bacterial FAD- and FMN-containing hemoprotein oxygenases or reductases (32, 33).

Analysis of FAD and FMN content of NO synthase suggests that two molecules of FAD and only one molecule of FMN are bound noncovalently per functional dimer. It is possible that FMN binds less tightly and is partially lost during the purification procedure. Alternatively, the 2:1:1 stoichiometry of FAD/FMN/NO synthase dimer could be due to differential flavin binding by subunits of the enzyme. Some form of heterogeneity is also suggested by the observed

multiple banding between 125 and 135 kDa in silver-stained SDS/polyacrylamide gels. This may reflect differential cofactor binding, posttranslational modification, or merely a minor degree of proteolysis.

Constitutive NO synthase appears to provide critical homeostatic and regulatory functions in the cardiovascular and nervous systems. Inducible NO synthase, on the other hand, has been implicated in several pathological states, including septic shock, vascular leak syndrome associated with cytokine therapy, uremia, and diabetes (11, 12, 34, 35). Therapeutic inhibition of inducible NO synthase should be sufficiently specific not to interfere with the constitutive enzyme. The purification of the inducible NO synthase provides an opportunity to investigate both its unusual biochemistry and a means for its selective inhibition.

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1. Ignarro, L. J. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 535–560.
2. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J. C., Amber, I. J. & Lancaster, J. R. (1990) in *Nitric Oxide from L-Arginine: A Bioregulatory System*, eds. Moncada, S. & Higgs, E. A. (Elsevier, New York), pp. 189–223.
3. Nathan, C. F. & Hibbs, J. B., Jr. (1991) in *Current Opinion in Immunology*, eds. Silverstein, S. & Unkeless, J. (Current Science, London), Vol. 3, pp. 65–70.
4. Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 682–685.
5. Schmidt, H. H. H. W., Pollock, J. S., Nakane, M., Gorsky, L. D., Forstermann, U. & Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 365–369.
6. Mayer, B., John, M. & Bohme, E. (1991) *FEBS Lett.* **277,** 215–219.
7. Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K., Ohkawa, S., Ohnishi, K., Terao, S. & Kawai, C. (1991) *J. Biol. Chem.* **266,** 3369–3371.
8. Stuehr, D. J. & Marletta, M. A. (1987) *J. Immunol.* **139,** 518–525.
9. Drapier, J. C. & Hibbs, J. B., Jr. (1988) *J. Immunol.* **140,** 2829–2838.
10. Ding, A., Nathan, C. F. & Stuehr, D. J. (1988) *J. Immunol.* **141,** 2407–2412.
11. Kilbourn, R. G., Gross, S. S., Jubran, A., Adams, J., Griffith, O. W., Levi, R. & Lodato, R. F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3628–3632.
12. Kilbourn, R. G., Jubran, A., Gross, S. S., Griffith, O. W., Levi, R., Adams, J. & Lodato, R. F. (1990) *Biochem. Biophys. Res. Commun.* **172,** 1132–1138.
13. Bredt, D. S., Hwang, P. M. & Snyder, S. H. (1990) *Nature (London)* **347,** 768–770.
14. Kwon, N. S., Nathan, C. F. & Stuehr, D. J. (1989) *J. Biol. Chem.* **264,** 20496–20501.
15. Tayeh, M. A. & Marletta, M. A. (1989) *J. Biol. Chem.* **264,** 19654–19658.
16. Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E. & Stuehr, D. J. (1990) *J. Biol. Chem.* **265,** 13442–13445.
17. Stuehr, D. J., Kwon, N. S. & Nathan, C. F. (1990) *Biochem. Biophys. Res. Commun.* **168,** 558–565.
18. Stuehr, D. J., Fasehun, O. A., Kwon, N. S., Gross, S. S., Gonzalez, J. A., Levi, R. & Nathan, C. F. (1991) *FASEB J.* **5,** 98–103.
19. Stuehr, D. J. & Griffith, O. W. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol.*, in press.
20. Feelisch, M. & Noak, E. A. (1987) *Eur. J. Pharmacol.* **139,** 19–30.
21. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L. & Wiseman, J. (1991) *J. Biol. Chem.* **266,** 6259–6263.
22. Gnanaiah, W. & Omdahl, J. L. (1986) *J. Biol. Chem.* **261,** 12649–12654.
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
24. Stuehr, D. J., Kwon, N. S., Gross, S. S., Thiel, B. A., Levi, R. & Nathan, C. F. (1989) *Biochem. Biophys. Res. Commun.* **161,** 420–426.
25. Billiar, T. R., Curran, R. D., Stuehr, D. J., Stadler, J., Simmons, R. L. & Murray, S. A. (1990) *Biochem. Biophys. Res. Commun.* **168,** 1034–1040.
26. Kilbourn, R. G. & Belloni, P. (1990) *J. Natl. Cancer Inst.* **82,** 772–776.
27. Werner-Felmayer, G., Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G. & Wachter, H. (1990) *J. Exp. Med.* **172,** 1599–1607.
28. Beasly, D., Schwartz, J. H. & Brenner, B. M. (1991) *J. Clin. Invest.* **87,** 602–608.
29. Iyanagi, T. & Mason, H. S. (1973) *Biochemistry* **12,** 2297–2308.
30. Vermilion, J. L., Ballou, D. P., Massey, V. & Coon, M. J. (1981) *J. Biol. Chem.* **256,** 266–267.
31. Otvos, J. D., Krum, D. P. & Masters, B. S. S. (1986) *Biochemistry* **25,** 7220–7228.
32. Narhi, L. O. & Fulco, A. J. (1986) *J. Biol. Chem.* **261,** 7160–7169.
33. Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M. & Kredich, N. M. (1989) *J. Biol. Chem.* **264,** 15796–15808.
34. Remuzzi, G., Perico, N., Zoja, C., Corna, D., Macconi, D. & Vigano, G. (1990) *J. Clin. Invest.* **86,** 1768–1771.
35. Southern, C., Schulster, D. & Green, I. C. (1990) *FEBS Lett.* **276,** 42–44.